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Amino Acid Conferred Protection against Melphalan: Interference with Leucine Protection of Melphalan Cytotoxicity by the Basic Amino Acids in Cultured Murine L1210 Leukemia Cells

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SUMMARY

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Melphalan cytotoxicity to murine L1210 leukemia cells in culture is influenced by the amino acid composition of the incubation medium. Deletion of glutamine or leucine from RPMI 1630 medium increased the cytotoxicity produced during a 35 min exposure of L1210 cells to melphalan by 10-fold and 2-fold, respectively, indicating that a substantial proportion of the protection afforded L1210 cells from melphalan cytotoxicity in an amino acid environment can be attributed to these amino acids. However, protection from melphalan cytotoxicity in cells first incubated with leucine in a balanced salt solution containing bovine serum albumin and glucose was 10 times better than that in cells incubated with leucine in RPMI 1630 medium containing all amino acids except glutamine. This difference was due to an increase in the accumulation of intracellular melphalan in medium containing leucine and the basic amino acids. The increases in intracellular drug and resulting cytotoxicity decrease with increasing carbon chain length within the arginine homologous series (α-amino-γ-guanidinobutyric acid > arginine > homoarginine). However, the lowest arginine homologue, α-amino-β-guanidinopropionic acid, was ineffective. Maximum promotion of melphalan cytotoxicity by α-amino-γ-guanidinobutyric acid occurred at concentrations equimolar with leucine and was not substantially influenced by the length of prior incubation with the homologue. Interaction between melphalan and the basic amino acids with the leucine-preferring transport system in L1210 cells plays a significant role in melphalan cytotoxicity.

INTRODUCTION

Melphalan[2] cytotoxicity to L1210 murine leukemia cells in culture is reduced in growth medium containing amino acids (1). Leucine and glutamine were found to be primarily responsible for this decrease in melphalan cytotoxicity under conditions in which cells had been incubated with individual amino acids in Dulbecco's phosphate buffered saline (PBS)[3] containing 0.1 mM

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[2] Melphalan is an acronym for L-phenylalanine mustard, which is frequently abbreviated L-PAM.

[3] The abbreviations used are: PBS, Dulbecco's phosphate buffered saline (pH 7.4); BSA, bovine serum albumin; PAG, Dulbecco's phosphate buffered saline containing 0.1 mM bovine serum albumin and 0.25% glucose (pH 7.4).

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bovine serum albumin (BSA) and then given a 35 min exposure to the alkylating agent (1). Reduction in cytotoxicity is accounted for by a corresponding reduction in melphalan transport (2, 3). Kinetic analysis indicated that melphalan transport occurs via a high-affinity amino acid transport system of the leucine (L) type and that the protection by leucine can be attributed to its higher affinity for this carrier (3). However, protection of cells from melphalan cytotoxicity by leucine in complete culture medium is less than that obtained in a balanced salt solution containing albumin and glucose. This prompted additional investigation on the factor interfering with leucine protection. In this report we present evidence that an interaction between melphalan, cationic amino acids, and substrates of the leucine-preferring transport system is responsible for this effect.

MATERIALS AND METHODS

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories, Inc., Elkhart, Indiana. Fetal calf serum was purchased from Flow Laboratories, Rockville, Maryland; RPMI 1630 medium, fully supplemented with amino acids or with leucine and/or glutamine omitted and PBS were supplied by the NIH Media Unit. Gentamicin (Schering, 50 mg/ml) and fungizone (250 μg/ml) were purchased from Microbiological Associates, Bethesda, Maryland, and Grand Island Biological Co., Grand Island, New York, respectively. All amino acids were of the L-configuration and were obtained from Calbiochem, San Diego, California, with the exception of L-α-amino-β-guanidinopropionic acid, which was purchased from Pierce Chemical Co., St. Louis, Missouri. The silicone oil, Versilube F-50 (specific gravity 1.045 at 25°; viscosity 70 centistokes at 25°) was obtained from Harwick Chemical Corp., Cambridge, Mass. Unlabeled melphalan was purchased from Burroughs Wellcome Co., Research Triangle Park, North Carolina.

Melphalan (6.4 mCi/mmole), labeled in the chloroethyl groups with ^{14}C, was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute, Menlo Park, Calif. Radiochemical purity was 97% as determined by thin layer chromatography on silica gel in n-butanol-acetic acid-water (7:2:1).

Unlabeled and labeled melphalan was prepared fresh daily in 75% ethyl alcohol containing an equimolar concentration of hydrochloric acid. Further dilutions of melphalan were made in aqueous medium prior to use in order to minimize hydrolysis. Labeled and unlabeled melphalan had the same transport characteristics as determined by isotope dilution and the same cytotoxic potency to L1210 cells as determined by clonal growth (1, 4).

Cytotoxicity assays. The conditions for maintenance of cell cultures and for exposure of cells to melphalan have been described (1). Modifications included the use of cells in the logarithmic growth phase ($5–10 \times 10^5$ cells/ml), incorporation of fungizone (0.25 μg/ml) in the maintenance medium and 0.25% glucose in the standard incubation medium of PBS and 0.1 mM BSA (PAG).

A minimal LD_{100} concentration of melphalan was used for appropriate studies described in the text. This concentration was defined (1) as the lowest concentration of melphalan which results in 100% mortality after incubation with cells for 35 min in PAG and was used to minimize experimental variation due to the sharp dose response curve of the drug. This drug dose is 6.5 μM for a cell concentration of 10^5 cells/ml and 7.0 μM for a cell concentration of 10^6 cells/ml.

Cells were harvested after a 35 min exposure to melphalan or the solvent, ethanol, washed twice in RPMI 1630 medium supplemented with 20% fetal calf serum, and suspended in the same medium at $1.0–1.2 \times 10^5$ cells/ml. The cytotoxicity of melphalan was assessed by clonal growth of surviving cells according to the procedure of Chu and Fischer (4) with minor modifications (1).

Melphalan uptake studies. Logarithmic phase L1210 cells ($5–10 \times 10^5$ cells/ml) were harvested by centrifugation at $300 \times g$ for 5 min, washed three times in transport medium, composed of PBS containing 0.1 mM BSA and 0.1% glucose and were then suspended at 2.0×10^6 cells/ml in the same buffer system. Cells were maintained at 37° during all phases of the uptake study, and

all experiments were completed within 1 hr of removal of the cells from growth medium. Such cells were found to be 90–100% viable, as determined by clonal growth.

Cells (2.0×10^6 cells/ml) were added to the appropriate volume of transport medium with or without amino acid and the uptake of melphalan was initiated by addition of labeled drug, as indicated in the individual experiments. Aliquots of the incubation mixture were layered on Versilube F-50 silicone oil in a microcentrifuge tube, and uptake was terminated by centrifugation of the cells through the oil at 12,000 × *g* for 1 min in an Eppendorf microcentrifuge. Individual uptake estimates were performed in triplicate, and cell recovery was found to be greater than 99%. Tips containing the cell pellet were cut off, the cell pellets were solubilized in 0.2 N NaOH, liquid scintillation fluor was added and melphalan uptake estimated in a Packard liquid scintillation counter.

Nonspecific adsorption of labeled melphalan was estimated by measuring drug binding at 0–4° following exposure of cells to 6.5 μM melphalan for time periods up to 24 min. Since the amount of drug bound (<0.3 pmoles per 10^5 cells) was negligible with respect to the amount transported, no correction factor was applied. Data are corrected for trapped extracellular drug (0.04 pmole per 10^5 cells). Intracellular radioactivity was identified as unhydrolyzed melphalan by its R_f of 0.50, following thin layer chromatography of the cell extract on silica gel 60 with n-butanol : acetic acid : water (4:1:1) (5).

RESULTS

Melphalan cytotoxicity in medium containing amino acids. The amino acid composition of the incubation medium determined the degree of melphalan cytotoxicity to L1210 murine leukemia cells, and substantial protection was provided by leucine and glutamine (Table 1). However, the extent of protection by leucine in tissue culture medium was one tenth that provided by this amino acid in PBS containing only BSA and glucose. This was in contrast with only a 40% reduction in the protection provided by glutamine in tissue culture medium (Table 1) and was the basis for further inquiry to determine which amino acids interfered with leucine protection.

TABLE 1

Melphalan cytotoxicity in media containing amino acids

Logarithmic phase L1210 cells ($5–10 \times 10^5$ cells/ml) were harvested from growth medium and washed three times at 37° in either PBS containing 0.1 mM BSA and 0.25% glucose (PAG), RPMI 1630, or in RPMI 1630 lacking leucine, glutamine or both, and supplemented with 0.1 mM BSA. Cells (1.0×10^5 cells/ml) were then incubated in the appropriate medium for 35 min in the presence of melphalan (2.0 μg/ml), a minimal LD_{100} dose. When melphalan was omitted, an equivalent volume of the solvent, ethanol, was added. Cultures were harvested and prepared for clonal growth in soft agar. Data are expressed as the number of colonies (mean ± S.D., N = 2) observed after 14 days of clonal growth.

Medium variation	Cells forming colonies per ml
A. Melphalan omitted	100,000
B. Effect of addition of amino acids	
PAG	0
PAG + 0.34 mM leucine	37,000 ± 3,000
PAG + 1.85 mM glutamine	2,500 ± 500
C. Effect of deletion of amino acids	
RPMI 1630	39,700 ± 4,500
Deletion of leucine (0.34 mM)	23,700 ± 1,700
Deletion of glutamine (1.85 mM)	3,900 ± 2,100
Deletion of leucine and glutamine	2,200 ± 770

Interference with leucine protection against melphalan cytotoxicity. The basic amino acids promoted melphalan cytotoxicity in leucine-protected cells incubated in PBS containing BSA and glucose (Table 2). Lysine and arginine were effective promoters of melphalan cytotoxicity, but histidine was ineffective. Arginine was the most effective of all natural amino acids examined and resulted in a 5-fold reduction in colony formation (Table 2).

Structure-activity relationship of homologues of arginine. Melphalan cytotoxicity to leucine-protected cells increased with decreasing carbon chain length of the arginine homologous series, α-amino-γ-guanidinobutyric acid > arginine > homoarginine (Table 3). However, the lowest homologue

TABLE 2

Promotion of melphalan cytotoxicity in leucine-protected cells by the basic amino acids

Logarithmic phase L1210 cells (5–10 × 10^5 cells/ml) were harvested from growth medium and washed three times in PAG at 37°. Cells (1.0 × 10^5 cells/ml) were then incubated for 15 min in PAG containing 0.1 mM leucine or in PAG containing 0.1 mM leucine and the desired basic amino acid at 0.1 mM. Melphalan (1.5 μg/ml), a minimal LD_{100} dose, or an equivalent volume of ethanol was then added and the incubation continued for an additional 35 min. At that time melphalan toxicity was reduced by addition of leucine to give a final concentration of 2 mM and the cells were prepared for clonal growth in soft agar. Data are expressed as the number of colonies (mean ± S.D., N = 3) observed after 14 days of clonal growth.

The following L-amino acids did not promote melphalan cytotoxicity in leucine-protected L1210 cells: alanine, asparagine, aspartic acid, cystine, glutamic acid, glycine, hydroxyproline, isoleucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine.

Medium variation	Cells forming colonies per ml
A. Melphalan omitted	100,000
B. PAG	0
C. PAG + 0.1 mM leucine	83,000 ± 1,500
+ histidine	80,000 ± 3,000
+ lysine	25,000 ± 800
+ arginine	16,600 ± 4,500

of arginine, α-amino-β-guanidinopropionic acid, was ineffective in promoting melphalan cytotoxicity in leucine-protected cells, as was γ-guanidinobutyric acid, the counterpart of α-amino-γ-guanidinobutyric acid lacking an α-amino group.

Arginine and its homologues increase intracellular melphalan. Promotion of melphalan cytotoxicity in leucine-protected cells by arginine and its homologues was accompanied by a corresponding increase in intracellular melphalan (Fig. 1). The small increases in intracellular drug provided by arginine and its homologues resulted in large increases in cytotoxicity (Table 3). These results are in agreement with our earlier observations that cytotoxicity ranged from minimal to essentially complete within a small critical range of net melphalan uptake of 2 to 5 pmoles per 10^5 cells (3).

In order to obtain an understanding of the interaction between α-amino-γ-guanidinobutyric acid and melphalan with the leucine-preferring transport system, studies were undertaken to determine whether leucine was required for the increase in intracellular melphalan provided by this lower homologue of arginine. The results indicated that α-amino-γ-guanidinobutyric acid

TABLE 3

Promotion of melphalan cytotoxicity in leucine-protected cells by arginine and its homologues

Logarithmic phase L1210 cells (5–10 × 10^5 cells/ml) were harvested from growth medium and washed three times in PAG at 37°. Cells (1.0 × 10^5 cells/ml) were then incubated for 15 min in PAG, in PAG containing 0.1 mM leucine, or in PAG containing arginine or its homologue at 0.1 mM, either without or with leucine as indicated. Melphalan (1.5 μg/ml), a minimal LD_{100} dose, or an equivalent volume of ethanol was then added and the incubation continued for an additional 35 min. At that time melphalan toxicity was reduced by addition of leucine to give a final concentration of 2 mM and the cells were prepared for clonal growth in soft agar. Data are expressed as the number of colonies (mean ± S.D., n = 2) observed after 14 days of clonal growth.

Medium	Addition (0.1 mM)	Cells forming colonies per ml
A. Melphalan omitted	None, homoarginine, arginine or α-amino-γ-guanidinobutyric acid	100,000
B. PAG	None	0
C. PAG	Homoarginine, arginine or α-amino-γ-guanidinobutyric acid	0
D. PAG + 0.1 mM leucine	None	82,000 ± 3,000
PAG + 0.1 mM leucine	Homoarginine	21,700 ± 400
PAG + 0.1 mM leucine	Arginine	14,100 ± 1,800
PAG + 0.1 mM leucine	α-amino-γ-guanidinobutyric acid	9,800 ± 2,100
PAG + 0.1 mM leucine	α-amino-β-guanidinopropionic acid	81,000 ± 2,500
PAG + 0.1 mM leucine	γ-guanidinobutyric acid	82,000 ± 3,000

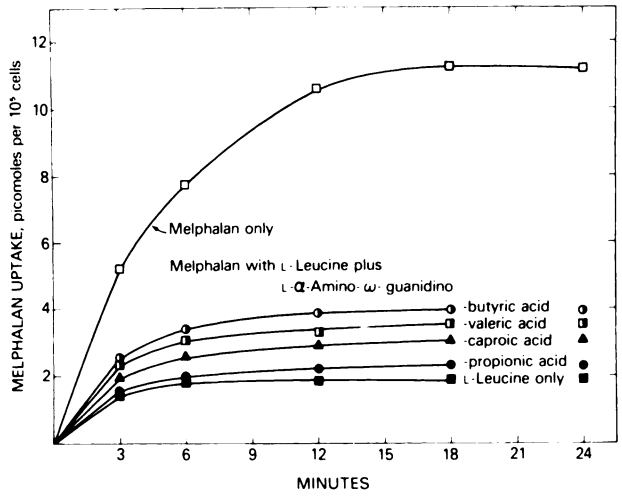

FIG. 1. *Promotion of melphalan uptake in leucine-protected cells by arginine and its homologues.*

Logarithmic phase L1210 cells (5–10 × 10^5 cells/ml) were harvested from growth medium, washed 3 times in transport medium at 37° and suspended at 2.0 × 10^6 cells/ml. Cells (1.33 × 10^6 cells/ml) were then incubated for 15 min at 37° in the same medium with shaking under one of the following experimental conditions: no amino acid supplementation, □; with 0.1 mM leucine, ■; with 0.1 mM each of leucine and α-amino-β-guanidinopropionic acid, ●; with 0.1 mM each of leucine and homoarginine, ▲; with 0.1 mM each of leucine and arginine, ◨; or with 0.1 mM each of leucine and α-amino-γ-guanidinobutyric acid, ◑. [^{14}C]Melphalan was then added to yield a final concentration of 6.5 μM. Triplicate 200 μl aliquots were removed at appropriate times and melphalan uptake was terminated by centrifugation of cells through silicone oil at 12,000 × *g*.

by itself did not increase intracellular drug but slightly reduced drug uptake (Fig. 2). In the presence of valine, a nonprotective amino acid (1) that is partially transported by the leucine-preferring transport system in L1210 cells (3), α-amino-γ-guanidinobutyric acid promoted melphalan uptake slightly (Fig. 2).

Concentration and time dependence of α-amino-γ-guanidinobutyric acid promotion of melphalan cytotoxicity in leucine-protected cells. Promotion of melphalan cytotoxicity by α-amino-γ-guanidinobutyric acid is concentration dependent (Fig. 3). Maximum promotion of cytotoxicity occurred at a concentration equimolar with leucine (100 μM) and resulted in a 1 log decrease in the surviving fraction.

The extent of prior incubation with α-amino-γ-guanidinobutyric acid did not alter the degree of promotion of melphalan cytotoxicity in leucine-protected cells (Table 4). No difference in colony formation was found when cells were first incubated with α-amino-γ-guanidinobutyric acid for time periods from 2.5–20 min. This indicates the rapidity with which the homologue can increase intracellular levels of melphalan in the presence of a competitive substrate such as leucine. A slight reduction in the

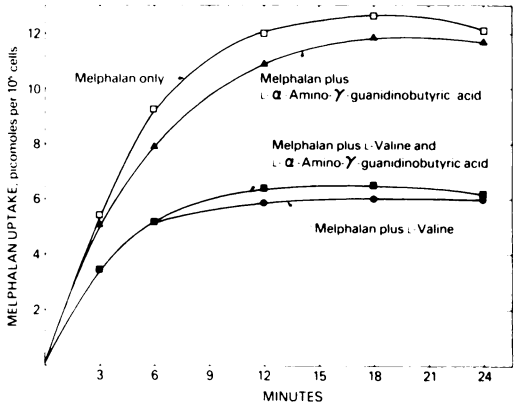

FIG. 2. *Effect of valine on melphalan uptake in the presence of α-amino-γ-guanidinobutyric acid.*

Logarithmic phase L1210 cells were prepared and incubated as described in the legend of Figure 1 under the following conditions: no amino acid supplementation, □; 0.1 mM α-amino-γ-guanidinobutyric acid, ▲; 0.1 mM valine, ●; or 0.1 mM each of valine and α-amino-γ-guanidinobutyric acid, ■. [^{14}C]Melphalan was then added to yield a concentration of 6.5 μM and the experiment was continued as described in the legend for Fig. 1.

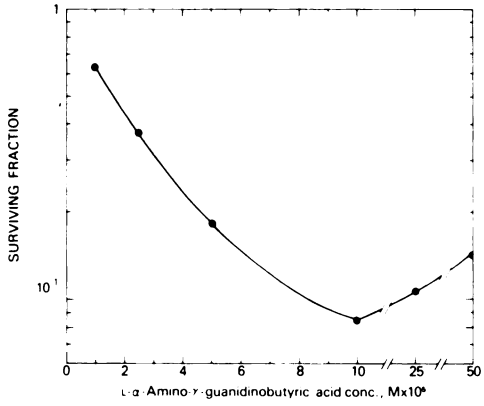

FIG. 3. *α-amino-γ-guanidinobutyric acid concentration and melphalan cytotoxicity in leucine-protected cells.*

Logarithmic phase L1210 cells were prepared in PAG as described in the legend to Fig. 1. Cells (1.0×10^5 cells/ml) were then incubated for 15 min in PAG containing 0.1 mM leucine and the appropriate concentration of α-amino-γ-guanidinobutyric acid. Melphalan (1.5 μg/ml), a minimal LD_{100} dose, was then added, and the incubation continued for an additional 35 min. At that time melphalan toxicity was reduced by addition of leucine to give a final concentration of 2 mM, and the cells were prepared for clonal growth in soft agar. Data are expressed as the number of colonies observed after 14 days of clonal growth. Prior incubation of cultures with 0.1 mM, 0.25 mM, and 0.5 mM α-amino-γ-guanidinobutyric acid for 15 min served as controls to discern whether this amino acid reduced melphalan cytotoxicity in the absence of added leucine. No reduction in melphalan cytotoxicity was found in the absence of added leucine at concentrations of 0.25 mM or lower.

degree of promotion of melphalan cytotoxicity occurred when melphalan was added together with the arginine homologue.

DISCUSSION

The transport of neutral amino acids in the Ehrlich ascites tumor cell occurs by overlapping transport systems. The first, designated system L (6, 7), is sodium independent and generally is responsible for the transport of amino acids with lipophilic side chains such as leucine. The second, system A, exhibits dependence on sodium and is responsible for the transport of straight chained aliphatic amino acids such as alanine. A system, designated ASC, exhibits sodium dependency and is responsible for the transport of 3 and 4 carbon aliphatic, hydroxyaliphatic and sulphur containing amino acids (8), and another designated by Ly^+, is responsible for the transport of basic amino acids (9). Considerable overlapping and interaction occur between these systems.

It has been demonstrated that, in the Ehrlich ascites tumor cell, neutral amino acids substantially decrease cationic amino acid transport (9, 10) whereas cationic amino acids only slightly decrease the transport of neutral amino acids. The results described in the present study may parallel those described for lysine homologues (10) in which the inhibitory effect of these homologues (lysine > ornithine >

TABLE 4

Time of incubation with α-amino-γ-guanidinobutyric acid and melphalan cytotoxicity in leucine-protected cells

Logarithmic phase L1210 cells ($5–10 \times 10^5$ cells/ml) were harvested from growth medium and washed three times in PAG at 37°. Cells (1.0×10^5 cells/ml) were then incubated for 15 min in PAG or in PAG containing 0.1 mM leucine. Melphalan (1.5 μg/ml), a minimal LD_{100} dose, or an equivalent volume of ethanol was added either with α-amino-γ-guanidinobutyric acid (0.1 mM) or following incubation of the cells with this cationic amino acid for an additional time period as indicated and the incubation continued for 35 min. At that time melphalan toxicity was reduced by addition of leucine to give a final concentration of 2 mM and the cells were prepared for clonal growth in soft agar. Data are expressed as the number of colonies (mean ± S.D., $n = 2$) observed after 14 days of clonal growth.

Medium	Minutes incubation with α-amino-γ-guanidinobutyric acid	Cells forming colonies per ml
A. Melphalan omitted	—	100,000
B. PAG	—	0
C. PAG + 0.1 mM leucine	—	82,000 ± 3,000
PAG + 0.1 mM leucine	0	13,400 ± 2,500
PAG + 0.1 mM leucine	2.5–20	10,000 ± 2,000

α,γ-diaminobutyric acid > α,β-diaminopropionic acid) upon basic amino acid transport decreases with decreasing carbon chain length. Such reduced interaction of short-chained basic amino acids such as α,γ-diaminobutyric acid and α,β-diaminopropionic acid with the cationic amino acid transport system has been correlated with increased reactivity with system L (11).

Melphalan uptake and cytotoxicity can serve as an indicator of the complex interactions in the transport of the natural amino acids. Such an interaction between the leucine-preferring pathway (L system), which is responsible for both leucine and melphalan transport (2, 3), and a cationic amino acid transport pathway (Ly^+ system) has been studied by Christensen, Handlogten, and Thomas in Ehrlich ascites cells and rabbit reticulocytes (9). Such cationic amino acids stimulated the uptake of L system amino acids by 15–40% by exchange diffusion, which involved equivalent efflux of the cationic amino acid from the cell. Such exchange diffusion was found to be concentration dependent, and could only be observed at higher concentrations of the neutral amino acids (5 mM or higher). This may provide an explanation of why α-amino-γ-guanidinobutyric acid did not stimulate melphalan uptake in the absence of added leucine, although the precise mechanism by which the basic amino acids increase intracellular melphalan will require further study. Leucine, a competitive substrate for melphalan transport by the L system, may become a co-substrate, thereby furnishing the essential concentration for an L system amino acid to participate in exchange diffusion with a cationic amino acid.

The results described in the present study raised the possibility that α-amino-γ-guanidinobutyric acid may improve melphalan therapy. However, intraperitoneal administration of the arginine homologue with melphalan did not improve melphalan therapy of intraperitoneally implanted L1210 treated 24 hours after tumor inoculation (12). Additional studies are being undertaken to determine its usefulness in an advanced L1210 tumor model. The latter is accompanied by a pronounced ascites in which the presence of amino acids may significantly alter the therapeutic response.

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